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AMS in Phytonutrition

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AMS in Phytonutrition

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Introduction

> *"Let food be our medicine and medicine our food"* - Hippocrate (4th century B.C.), the father of medicine

Food as medicine – the notion is as relevant in our present age of phytonutrition, genetically engineered foods, and an increasing population of health-conscious consumers, as it was in the age of Hippocrate several millennia back. Popular conceptions of foods and plants, however, have transformed substantially over the centuries. Presently, plants are esteemed for their reputed health benefits and we speak of them using the lexicon of modern chemistry. In medieval times, though, plants were considered the residences of divinities or malevolent spiritual forces. An example of one plant's conceptual evolution is illustrated by the tomato plant. Today, a tomato is nearly synonymous with its bright colorant lycopene, the hydrocarbon plant pigment responsible for its red color and putative enemy of cancer. In medieval Northern Europe, reference to what was then called a tomato, would not imply any medicinal benefits, but more likely suggest images of witches and werewolves (Figure 1). This fearful perception arose from the morphological resemblance of the tomato plant to poisonous members of the Solanceae family (particularly Belladonna) which were common "hexing herbs" of medieval witches. Old German folklore describes witches using plants of the nightshade family to evoke werewolves, a practice known as lycanthropy. This lore was clearly noted by Carl Linnaeus, the father of modern biological classification, when he named the tomato *Lycopersicon esculentum*, which translates to 'edible wolf peach', leading to the name of the phytonutrient—lycopene.

Figure 1. *Popular conceptions of foods have transformed substantially over the centuries. Now esteemed as a rich source of lycopene, to medieval Europeans the tomato had a baleful reputation*

Plants, and their intrinsic properties, have been the subjects of increasing attention over the past several decades. Today, plants are considered complex chemical factories that produce an array of biologically active phytochemicals for the consumer. Some of the more commonly known and studied compounds include the antioxidant flavonoids in diverse products such as tea and apple peels, high molecular weight tannins in berries and wine, or the isoflavones in soybean-derived products. The interest of health-conscious consumers and the mass media, has created a competition among the food and agricultural industries to create value-added food products enhanced or supplemented with phytochemicals. Creation of these products is possible through a combination of traditional plant breeding and genetic engineering (1-4). The introduction of genetically engineered foods (GE foods or genfoods) into the food and agricultural industries has sparked heated debate. Many concerns have been raised regarding the safety of genfoods foods, as well as the social, political and economic issues implicated with GE food production. As nutritionally enhanced products make the leap from laboratory to field, it will be the charge of the scientist to conclusively establish that any modifications in nutritional content have evident benefits.

Acute public interest in phytonutrition (the dietary use of phytochemicals found in plants for providing nutrients and optimal health) is propelled by the ongoing research of these microchemical food constituents as causative or preventative agents in disease. It is further supported by the findings in dietary research of people with higher fruit and vegetable intake demonstrating lower rates of cancer and cardiovascular disease (5). These conclusions have prompted hypotheses centered on antioxidants, including the well-known redox vitamins such as vitamin E and C, as well as non-essential components such as select carotenes and flavonoids. The positive association between fruits and vegetables and optimal health is popularly considered axiomatic. It is proven that dietary phytochemicals supply essential vitamins, minerals, and macronutrients to the consumer. It remains to be empirically established, however, if phytochemicals are indeed beneficial, or conversely, harmful, especially when given as high-dose supplements (6). Indeed, the notion that plant products are 'harmless' was disproved when chemoprevention studies found that ß-carotene can promote cancer in smokers (7, 8). These studies do not refute the beneficial role of phytochemicals in disease prevention, but they do pull favor towards a whole food-based approached for increasing photochemical intake and promoting good health.

A proven technique in pharmaceutical research, which is playing an integral role in phytonutrition, is mass spectrometry. Structural determination and identification are strengths of modern mass spectrometry. Moreover, hyphenated GC/MS (9), and LC/MS/MS (10-12) techniques are particularly well suited to establishing phytochemical content in plants, as well as the biological variance in human metabolism of phytochemicals, due to their high sensitivity, throughput, and ability to resolve overlapping components from complex matrices (13). However, elaborating the full complement of biological metabolites of phytochemicals at trace levels is a strength of radioisotopes, typically ^{14}C or ^{3}H. In this regard, ^{14}C-accelerator mass spectrometry (AMS) is affording new opportunities in the study of human phytochemical metabolism (14). AMS is a type of tandem isotope ratio mass spectrometer that measures the ratios of $^{14}C/C$ to parts per quadrillion or down to as few as 10^{5} ^{14}C atoms. Accordingly, attomolar concentrations (10^{-18}) of ^{14}C labeled chemicals are quantified in milligram-sized biological specimens. Labeled phytochemicals and their metabolites can be traced in unprocessed plasma, tissue, and excreta, without regard to compound stability or the efficiency of the extraction procedure, thus enabling balance studies of excreta. Although AMS involves

radioactivity, its high sensitivity reduces radiative exposure to negligible levels. Typical human doses range from 10-100 nanoCuries (0.37-3.7 kBq) of activity. Even at levels such as these, labeled nutrients can be traced for months in plasma and excreta. For establishing metabolite profiles, small (microliter) aliquots of minimally processed plasma or unprocessed urine are separated by liquid chromatographic systems prior to analysis. These radiochromatograms can serve as detectors for assessing the range of biotransformations and identifying target analytes. The low background of ^{14}C in the environment provides an unambiguous baseline for clearly identifying ^{14}C enriched fractions collected from chromatographic systems. Metabolite identity is then finalized by applying traditional MS and other techniques to concentrated HPLC fractions.

AMS is poised to assume a leading role in phytonutrition. For effective expansion of AMS into phytochemical investigations, suitably labeled substrates must be available. Many natural products found in foods, though, are difficult to synthesize due to complex stereochemistries and a multiplicity of polymeric forms. However, there are several options to overcome the aforementioned predicament. One option is the incorporation of a simple radiolabled precursor, perhaps ^{14}C-*para*-aminobenzoic acid (folate) or phenylalanine (catechin), through the plant's roots, or via leaves and stems by surface application. If the label is efficiently incorporated into the target, the food can conceivably be ingested intact to examine the effects of the plant matrix on the nutrient digestibility. Alternatively, plants can be grown in $^{14}CO_2$-enriched environments using atmospherically-isolated plant growth chambers. Photosynthetic labeling in the presence of $^{14}CO_2$ randomly labels the entire phytochemical constitution of the plant, thus creating a radiopharmacy of sorts. When following the appropriate fractionation, this yields a spectrum of compounds that can be used individually or possibly in synergistic combinations for *in vivo* and *in vitro* studies of human metabolism and catabolism.

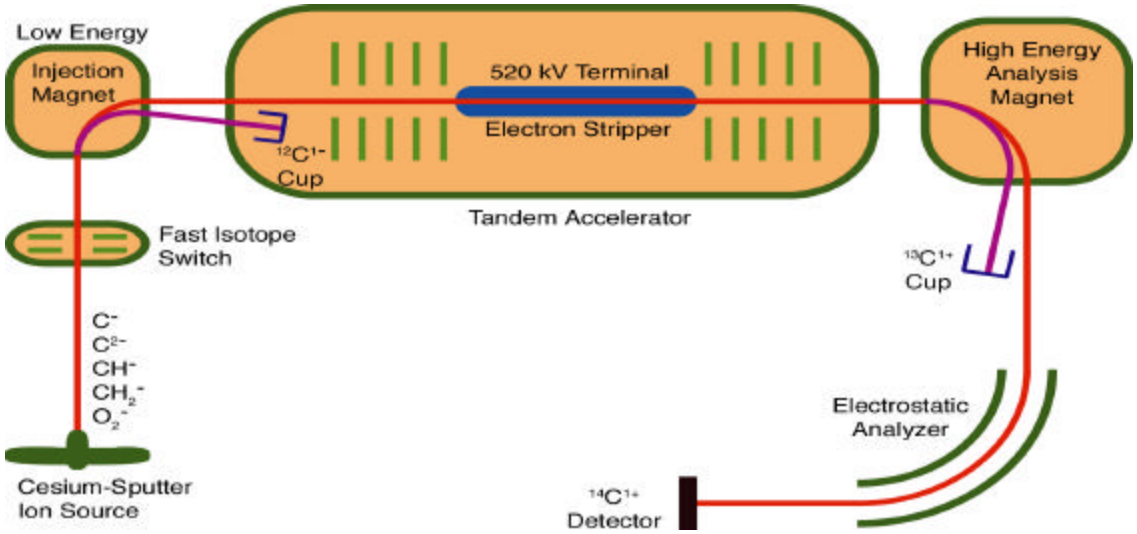
Throughout this presentation: *AMS in Phytonutrition*, a variety of MS Tools and isotope labels will be discussed. As suggested by its title, however, the presentation will focus chiefly on exploring on recent developments in the area of accelerator-based techniques, substantiated by the speaker's knowledge and research conducted at the University of California, Davis. To establish an appropriate context for this exploration, the technique of AMS and its history will be surveyed. A selection of investigative studies will then illustrate the unique capabilities AMS brings to the realms of biological tracing and metabolite profiling, as well as non-invasive assessments of phytonutrient metabolism and bioloabeling strategies. This presentation serves as an introduction to AMS and its developing role in phytochemical research. For further knowledge on the topic, several excellent reviews addressing the role of AMS in nutrition are available. (15, 16)

Overview of Accelerator Mass Spectrometry

In the 1940's Willard Libby at the Institute for Nuclear Studies developed the method of carbon dating using a highly sensitive Geiger counter. In this form of radiometric dating, the concentration of ^{14}C, the longest-lived radiosotope of carbon, records the time since the last carbon exchange with the atmosphere. The specific activity of the material thus serves as a reverse chronometer, accurate—but not highly so—to about to 60,000 years into the past.

Upon its development, radiocarbon dating had immediate and widespread consequences on many fields. However, the method of decay counting, as described by Libby, is an inefficient

means of quantifying the ^{14}C content of a sample since ^{14}C decays slowly: measuring 0.1% of the ^{14}C in a sample requires uninterrupted counting for 8.3 years {0.1% x 5730 years /ln 2} (By contrast, 0.1% of the population of ^{32}P is measured in 29.7 min). To overcome this limitation, very large quantities of sample, anywhere from grams to 100's of grams were required. More often than not, such quantities of material were either not available or could not be sacrificed to the measurement. In the late 1970's and throughout the '80's a mass spectrometric method for direct detection ^{14}C and other long-lived isotopes was developed in low-energy nuclear physics laboratories. The technique, accelerator mass spectrometry (AMS), although originally developed for the difficult task of radiocarbon dating, emerged in the 1990's as a useful bioanalytical tool for the quantification of ^{14}C and (other long-lived isotopes) in biochemical labeling and tracing.



Schematic of compact bio-AMS system at LLNL (Courtesy of Bruce Buchholz, LLNL)

Figure 2. *Biological samples for ^{14}C-AMS are typically combusted to CO_2 and then reduced to graphite. These samples are then bombarded by 3-10 keV Cs ions in a cesium-sputter ion source. This process physically knocks atoms and molecules out of the sample and contributes an electron to a fraction of the ejected particles, forming negative elemental or molecular ions. The production of negative ions removes the primary isobaric interference for radiocarbon, ^{14}N, since nitrogen does not form a stable negative ion. Singly ionized negative ions are accelerated to 40 kEV and then filtered by a low energy mass spectrometer that alternately switches between ions of mass about ^{14}C, i.e. ^{14}C, ^{13}CH, and $^{12}CH_2$ and mass 13, for the stable isotope measurements, into the accelerator. The field in the injection magnet is held constant and the isotopes are changed by quickly switching the energy (voltage) of the incoming ion beam. Negative ions are accelerated through 520 kV in the first stage of a tandem accelerator. At the end of this first acceleration stage these ions pass through an electron stripper, a small volume of ~45mT gas constrained by differential pumping. Here, particles undergo collisions, losing valence electrons. Depending on the accelerator potential, charge states of +1 to +4 are created. These positive ions then accelerate away from the positive potential to ground potential in the second half of the accelerator. The loss of electrons in the collision cells destroys all molecules, leaving only nuclear ions at relatively high energies (1.04 MeV for C). Positive ions exiting the accelerator are analyzed with a magnet and an electrostatic analyzer. An off-axis Faraday cup measures the $^{13}C^{1+}$ current after the analyzing magnet. A solid state particle detector counts individual $^{14}C^{1+}$ ions. The footprint of the compact Bio-AMS system is approximately 50 m^2.*

AMS is a type of tandem isotope ratio mass spectrometer that measures the ratios of $^{14}C/C$ to parts per quadrillion or down to as few as 10^5 ^{14}C atoms. In AMS, negative ions are generated in a cesium ion source and accelerated by a potential of 0.5-10 million volts. The accelerated ions are smashed through a thin carbon foil or inert gas that removes electrons and destroys all molecular species. After passing through a high-energy mass spectrometer and various filters, carbon nuclides are measured with current and particle detectors. A schematic of the compact bio-AMS instrument at Lawrence Livermore National Laboratories is shown in the adjoining figure (Figure 2) with a discussion of its mode of operation (14).

AMS provides the lowest detection limits of any isotopic measurement technique for not only ^{14}C, but also for other long-lived radionuclides that include ^{3}H, ^{10}Be, ^{26}Al,^{32}Si, ^{36}Cl, ^{41}Ca, ^{59}Ni, ^{99}Tc, ^{129}I, and several actinide isotopes. All isotopes for which AMS is developed have half-lives in the range of 10^3-10^7 years, with the exception of ^{3}H (half-life 12.25 years). On this basis, an AMS instrument could be described as an ultra sensitive scintillation counter. Such a description, however, is misleading for it suggests that the measurement is tied to the decay properties of the nuclide, which AMS is not. Radioactivity is not a requirement for AMS; rather, AMS profits from the extremely low natural abundance of radioisotopes. Stable isotopes occur at abundances of 0.1% to 50% of the natural element, whereas radioisotopes occur on Earth at parts per billion or less. For example, while ^{14}C is present at ~100 amol/mg of carbon in contemporary materials, the stable isotope of ^{13}C is present at levels around 1 micromol in the same amount of material. Quantifying on ^{14}C, therefore, extends the theoretical dynamic range of the measurement by a factor of 10^{-10}, translating into detection sensitivities of attomoles for ^{14}C in milligram-sized samples.

Most AMS systems operate at 3-10 MV and primarily measure natural radiocarbon. The larger systems analyze the elements important for geochronology, which typically require higher voltages. Conventional AMS systems are hardly "benchtop", or even laboratory-sized (15). The LLNL conventional AMS system, for example, fills a 550 m^2 area. This is a general-purpose research AMS system, however, that is being used for analyses from mass 3 to mass 240 (14). Radiocarbon dating with lower charge states has been demonstrated and several commercial spectrometers are becoming available, at varied size and cost, that will encourage widespread use in institutional and industrial research (17). Lawrenece Livermore also has a 1 MV BioAMS (described in Figure 2) dedicated to biochemical tracing of ^{3}H (18, 19) and ^{14}C analyses. A Single Stage AMS System (SSAMS) consisting of an open air 250 kV deck (not a tandem accelerator) is presently being marketed at a cost below $1 million.

Biological Tracing

The approach of phytochemical research can be considered comparable to that of pharmacokinetic studies. Pharmacokinetic data--absorption, distribution, metabolism, and excretion—is central to drug development and is attainable from the ability to measure drugs, at the concentration that they are found, in the human body during the course of therapy. Without the ability to execute such concentration measurements, pharmacokinetics could only be a theoretical exercise. This same statement holds true for phytochemical research. A bioactive phytochemical, in order to be an effective public health prophylactic, nutraceutical, or functional food component, must either be absorbed intact or as some active metabolite by most individuals and attain effective concentrations at the receptors or tissues. To draw conclusion, these concentration measurements must then be

associated to epidemiologic evidence, modulation of biomarkers, or some clinically defined endpoint. It merits noting, however, that phytochemical kinetic studies are technologically more challenging than similar drug studies in several ways. First, drugs are most often tested using large milligram to gram-sized doses, whereas phytochemicals are generally consumed in microgram to milligram concentrations. Secondly, pre-existing concentrations in tissues and fluids limit the ability to follow the fate of dietary constituents when given as a 'dose', either with food or as an isolate. These challenges can be surmounted through application of sensitive detection methods towards isotope labeled compounds that facilitate discrimination of exogenous intake from endogenous pools.

The application of AMS in phytochemical research is relatively new, however, isotopic labels have been a primary tool in tracing chemicals in natural systems for over 60 years (15, 20). The value of isotopes as elemental or molecular tracers depends on the detection methods: isotope ratio mass spectrometry (IRMS), nuclear magnetic resonance (NMR), decay production counting, AMS, etc... Short-lived radioactive isotopes have high sensitivity in decay counting above their very low backgrounds, but expose the participants to possible radiative harm. Tritium and radiocarbon, because of their availability, ease of detection by decay counting, and appropriateness to biologic questions, have been incorporated into human nutrition research. For example, radiotracer studies with ß-carotene conducted in the 1960's established that ß-carotene is first cleaved within the intestinal enterocyte to vitamin A active retinyl esters, then transported, via the lymphatics, into circulation (21). The full potential of radioisotopes has not been realized in regards to human nutrient metabolism due to health concerns over the use of ionizing radiation. In addition, practical material related to disposal cost has restricted widespread acceptance and application. In order to achieve adequate counting statistics with permissible blood sample, the use of radiotracers in many studies is not practical.

^{14}C-AMS was originally developed for, and remains most applied to, the area of carbon dating at levels of ratios of 14C/C at or below the contemporary levels of ^{14}C . The importance of ^{14}C as a biological tracer at concentrations above contemporary levels was not lost on the early developers of AMS for carbon dating. The possibility for the measurement of ^{14}C-glucose in humans was explored in the late 1970's at the University of Rochester (New York). This was one of two laboratories barely 100 miles apart that first published its methods of detecting natural ^{14}C at part-per-trillion levels using tandem Van de Graff accelerators (22). For all intents and purposes, AMS did not establish a foothold in bioanalytical tracing until AMS operations at Lawrence Livermore National Laboratory began in late 1988 (14). In the first study of its type, AMS was used to quantify the amount of meat carcinogen ^{14}C-MeIQX, covalently bound to mouse liver DNA (DNA adduct) following a very low-level dose (23-25). The first nutrients followed with the long-term biological tracing of folic acid in 1997 (26) and β-carotene in 2000 (27) at the University of California, Davis, in collaboration with LLNL. An important demonstration of AMS in human breath analysis came from Lund University in 1996 (28). The first use of AMS analysis in human mass balance and HPLC metabolite-profiling of a pharmaceutical appeared in 2002 at the CBAMS Institute (now Xcerleron) at York, United Kingdom (29).

Natural and synthetic a -tocopherols

Public interest in nutritional supplements has escalated dramatically over recent decades. To gain a market advantage, certain formulations are labeled as being more efficacious (biopotent) than competing products for reasons that are rarely scientifically based. For example, a consistently presented idea is that *'natural'*, meaning a plant derived concentrate, is superior to its *'synthetic'* alternative. Ultimately, the biopotency of one form over another needs to be determined on the basis of clinical and biochemcial endpoints (30). However, in lieu of these data, relative availabilities and retention are assessed using tracer studies that can record biological fate of the compound.

Natural and synthetic forms of α-tocopherol are available for use as vitamin E supplements and fortified foods. When discussing the biopotency of either form, it is necessary to carefully examine its correspondent data. Natural α-tocopherol is *RRR*-α-tocopherol, a pure stereoisomer. The conventional synthetic form, *all rac*-α-tocopherol (*all rac*), however, consists of equal amounts of 8 different stereoisomers (*RRR*-, *RRS*-, *RSR*-, *RSS*-, *SRR*-, *SSR*-, *SRS*-, *SSS*-) with only 1/8 the quantity represented as the natural *RRR* form. The long accepted ratio of biopotency of *natural* to *synthetic* α-tocopherol is 1.36 on the basis of an assay in pregnant rats. Such bioassays are not practical for humans, however, as they require condition of vitamin E deficiency. To assess the relative biopotency in humans, several authors have measured the relative plasma exposures of *natural* and *synthetic* α-tocopherol as a surrogate measure for biopotentcy using deuterium labels and GC/MS and LC/MS detectors (31-33). These data suggested a biopotency ratio closer to 2 than 1.36, and consequently, an adjustment in the official value has been proposed. The validity of this data has been questioned, though, for reasons that are both methodological and biological (30, 34). A primary concern centers around the use of pharmacological doses (15-150 milligrams) that do not mimic normal dietary intake patterns and overwhelm physiological transport systems (34). Indeed, capacity limitation of plasma vitamin E concentrations is likely mediated by the hepatic α-Tocopherol Transfer Protein (α-TTP), which is critical for biodiscrimination of vitamin E homologues and stereoisomers, as they exhibit differing affinities to the protein (35) Since binding to α-TTP is saturable and stereoselective, the pharmacological dosing approach may not be applicable for the assessment of vitamin E biopotency.

AMS sensitivity allows tracing nutrients at dietary relevant levels that do not saturate or perturb metabolic pathways. This model was perfectly suited, therefore, for a study of natural and synthetic forms of α-tocopherhol recently conducted at the University of California, Davis. The results are detailed in this presentation to highlight the unique capabilities that AMS brings to biochemical tracing rather than attempting to resolve the current dispute over the biopotency of α-tocopherols.

Plasma biokinetics

^{14}C-labeled tocopherols (natural and synthetic) were individually tested in a longitudinal design in a single male participant: The first dose consisted of *natural* α-tocopherol (^{14}C-*RRR* α-tocopherol 3.7 kBq or 100 nCi , 0.85 ug). Three months later, a similarly sized dose of the *synthetic* mixture was administered (^{14}C-*all rac* 3.7 kBq, 0.79 ug or 100 nCi). Both doses were given with cup of whole milk (10 g fat) to facilitate absorption. Frequent blood samples (5 ml) were taken via an indwelling venous catheter over the first two days after dosing. Less frequent samples were obtained via venipuncture over the course of 2 months post dosing. Twenty-five ul aliquots of plasma were

graphitized and carbon isotope ratio were measured to ±3% precision by AMS. Data are expressed as fmol of ^{14}C per ml of plasma; these values can easily be converted to labeled tocopherol equivalents using the specific activity of the labeled compounds.

The plasma concentration time course of the ^{14}C is shown in the adjoining figure for 63 days (Figure 3), with the 0-300 hr region expanded in the bottom panel. The doses were equal in terms of their ^{14}C content, and therefore, similar metabolism would yield highly equivalent

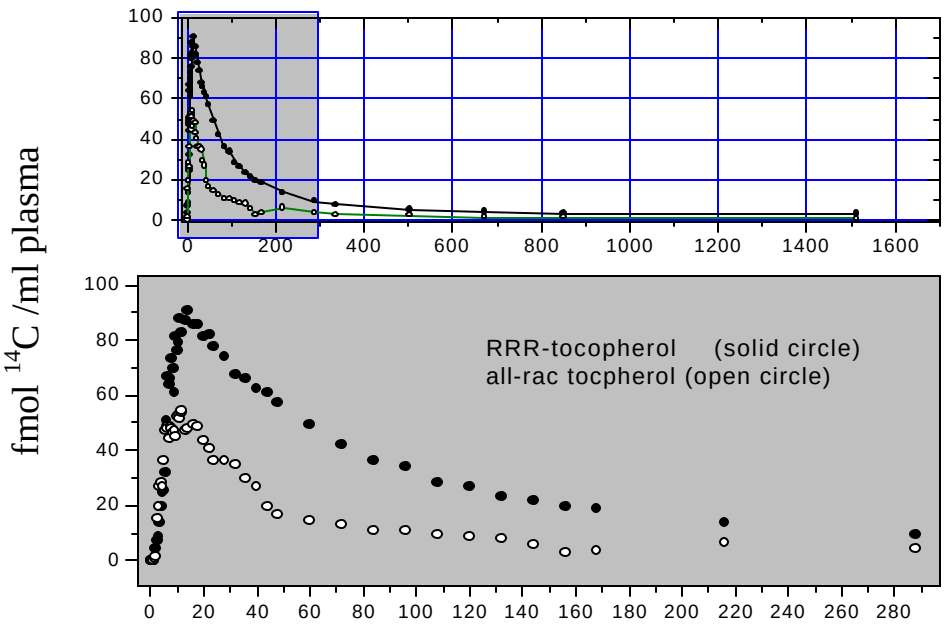


Figure 3*: Two ^{14}C doses were given in a longitudinal design in a single male volunteer: The first dose consisted of the single RRR isomer (RRR 3.7 kBq (100 nCi) , 0.85 ug). Three months later a similarly sized dose of the all-rac mixture was administered. Both doses were given with cup of whole milk (10 g fat) to facilitate absorption. Dose masses were 10,000 to 125,000 times less than similar studies using deuterium labeled isotopes with GC/MS or LC/MS detection.*

biokinetic patterns. For both compounds, there was a 2 hr lag in the appearance of activity, consistent with absorption with chylomicrons via the lymphatic system. An early appearance (30 min) could suggest alternative absorption in the portal blood, but this was not indicated. Concentrations of both forms rose equivalently until ~ 6 hr, suggesting an absence of biodiscrimination in the absorptive phase. Thereafter, the rate of rise slowed and concentrations vacillated. The increased complexity of this phase is attributed to continual inputs from the intestine, as well as resecretion of the tocopherols with hepatic lipoproteins (VLDL) and secretions from other tissues. Peak concentration occurred for both compounds between 12-14 hours, although concentrations close to the maximum were maintained over a time span from 10-24 hr. Concentrations did not begin to consistently decay until after the 24 hr time point. Post 24 hr, the decay of the *natural* α-tocopherol form was biphasic whereas the *synthetic* form displayed a complex decay pattern characterized by several resuspensions in concentration until settling into a terminal decay pattern after ~220 hr post dose administration.

Visual inspection of the concentration profiles reveals a muted plasma response for the *synthetic* relative to the *natural* α-tocopherol experiment: peak concentration in the 10-24 hr period for the *synthetic* were roughly 50% those of the *natural* form. This response cannot be attributed to differences in gut absorption since fecal measurements indicated similar digestibility (presented below). The magnitude of the differential response was confirmed by plotting cumulative total plasma exposure (Figure 4) for 10 days post dose. This plot clearly suggests the total availability of the *synthetic* dose relative to the *natural* dose, after 10 days is about 40%. Based on this information, the setting of the biopotency of *natural* compounds to twice that of the *synthetic* compounds would be supported (by this measure only) when using small, non-saturating doses.

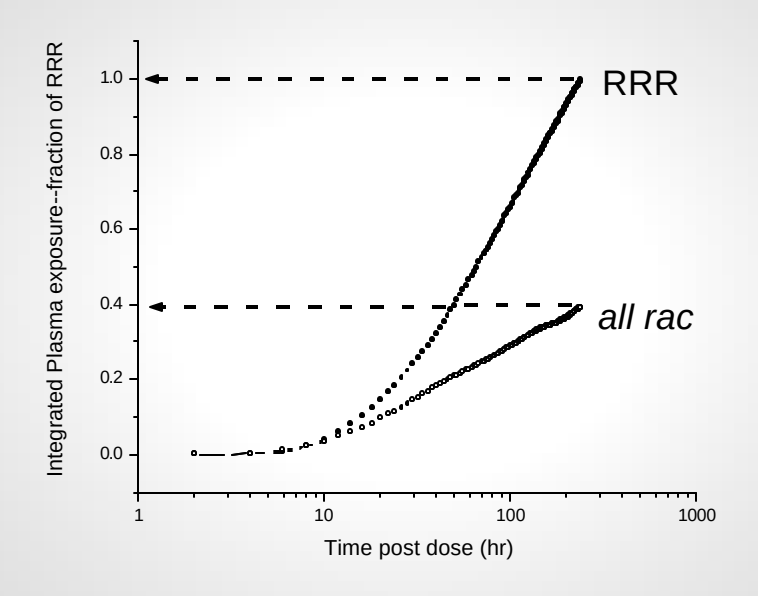


Figure 4*: Total plasma exposure to ^{14}C following administration of equivalent amount of natural (RRR) and synthetic (all rac)-a-tocopherol out to 250 hr post dose. A value of 1 is assigned to the RRR form. After 250 hr, the relative exposure to the synthetic form was about 40% that of the natural a-tocopherol.*

As demonstrated in this study, the small sample requirements for an AMS measurement (minimum requirements of one-mg or less of carbon or about 25 ul of plasma) provided for frequent blood sampling and a subsequent detailed temporal record of the kinetic behavior of the labeled tocopherols. The observed differences in the fine structure of the plasma concentrations can then be related to underlying mechanism, as well as provide basis for validation of existing theories—or development of new theories—for the biologic handling of these compounds. In this example, labeled compounds were not specifically speciated within biochemical subcompartments, ie lipoprotein carriers, incorporation of labeled red blood cells, or covalent binding to circulating albumin. This capability, though, has been demonstrated in other studies. The results of the aforementioned study were obtained via catheterization. It merits noting that such results might well have been obtained using microliter sized capillary finger sticks, similar to home health care testing kits used for the monitoring of blood glucose levels in diabetics. These techniques have dual advantages; they prevent the need for costly clinical support while lessening participant resistance associated with the discomfort experienced from venipuncture or catheterization.

Mass Balance: urine and feces

Classical balance methods, comparing compound intake with its total fecal excretion are considered challenging by traditional MS applications, as these methods require knowledge of the metabolite structures that would appear in the urine and stool. AMS sensitivity can reinvigorate classical balance studies using a radiolabel tracer at doses that will not saturate the binding capacity of the gastrointestinal system. Since AMS is a combustive process, performing balance measurements is relatively straightforward. Fecal samples are dispersed in alkaline solutions and subjected to a series of mechanical mixing and heating cycles before removing small aliquots for ^{14}C determinations. This process leads to a homogenous suspension from which representative samples can be removed for AMS analysis. Urine samples are analyzed either neat or subject to isotope dilution prior to graphitization using tributyrin as a carbon diluent.

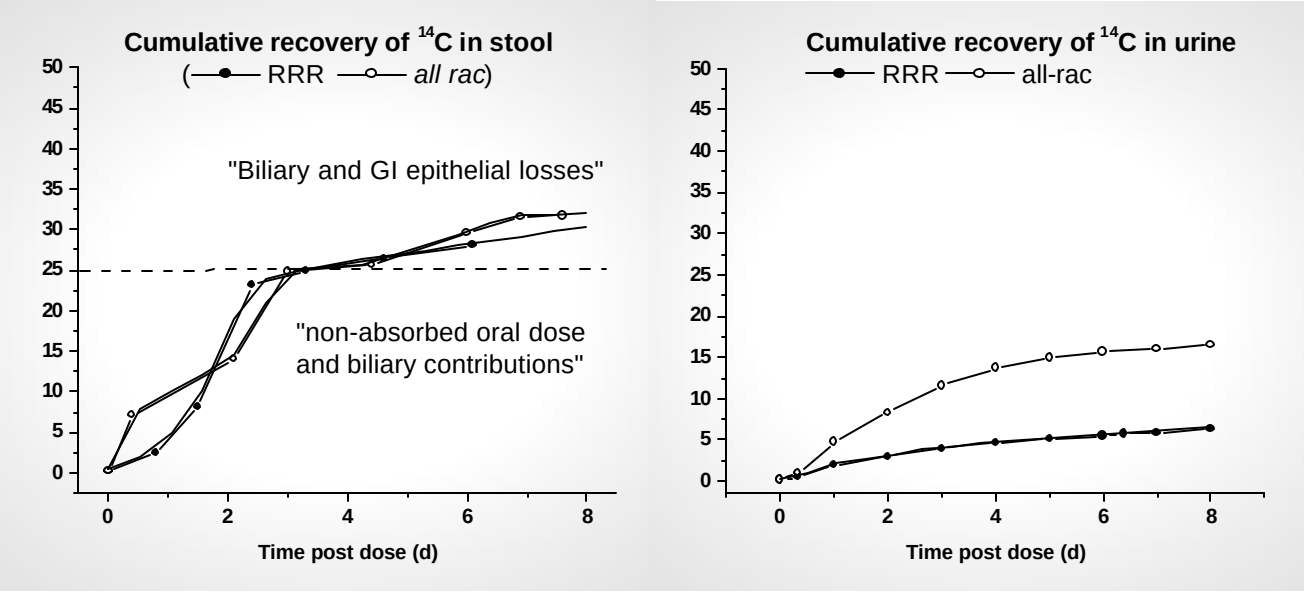


Figure 5*: Recovery of ^{14}C in urine (left) and stool following the two oral doses of labeled tocopherol. Intestinal absorption was equivalent for the two forms. Postabsorptively, the synthetic all-rac form underwent more rapid elimination via the urinary route. After 8 days, approximately 50% of the all rac, and 65% of the RRR were still present in the body.*

Cumulative urine and feces were collected for 8 days. The loss of label in the stool was biphasic, the transition between the phases occurring between 2 and 2.5 days (Figure 5). Early stool losses (< 3 days) are attributed to unabsorbed compound whereas later losses are attributed to gastrointestinal tract epithelial cell loss and inefficient recovery of tocopherols in the enterohepatic pool with bile loss. Losses were virtually equivalent for both compounds and accounted for 25% of the dose in this phase, suggesting at sub-physioloigcal doses the gut does not discriminate between the forms (32). Accordingly, the compounds were estimated to be

75% bioavailable. This approximation, however, does not take into account potential losses in the gut and other contribution of the rapidly re-excreted tocopherol and therefore may underestimate the true absorption.

Biodiscrimination between the two forms was not evident in the recovery of the label in the stool. The urine patterns, however, showed the loss of the *synthetic* form to be ~2.5-times that of the *natural* α-tocopherol form at 8 days post dose. This result is consistent with the observation that synthetic stereoisomers are less retained than the natural form, presumably due to differential affinities for hepatic binding proteins. Cumulative urine and fecal ^{14}C losses indicated after 8 days, approximately 50% of the *synthetic*, and 65% of the *natural* were still present in the body. The value shown here is considerably longer than the 2-3 day half-life of α-tocopherols which are typically reported. In fact, such a disparity is a recurrent observation in AMS-based studies. In other words, where dilution of the label into the background can occur only after several days in other protocols, AMS sensitivity affords the recording of true terminal elimination rates arising from the low seepage of retained compounds from tissues and organs.

Urinary metabolites: metabolite profiling

Isotope-labeled compounds reveal all of their metabolites in HPLC separation, but sensitivity when using LSC detection can be impacted by the need to keep chemical and radiative exposures low when using decay counting (36, 37). The small sample requirements and high sensitivity make AMS an excellent quantification tool for biochemical separatory processes that result in low concentration samples, such as urinary profiles.

Urine contains a myriad of information that can be sampled non-invasively. Vitamin E is extensively metabolized by side chain degradation initiated by cytochrome P450 (CYP)-mediated ? -hydroxylation of the phytyl side chain followed by ß-oxidation. These P450 enzymes are responsible for the metabolism of many drugs and other xenobiotics, and are likely of physiological importance in the postabsorptive regulation of tocopherol status in vivo (38-40). Accordingly, metabolite profiles may be useful in studying disease progression, effects of interventions (such as antioxidant supplements or drugs, or defining vitamin E requirements) (41, 42).

The first described urinary metabolites were identified by Simon *et al* (43) as α-tocopheronic acid and its lactone in the 1956. The eponymous 'Simon' metabolites, had an open chroman structure, consistent with α-tocopherol that had reacted as an antioxidant. In 1995, however, Schultz et al found the Simon metabolites to be mainly products of oxidative modification of a precursor, α-CEHC, during the analytical workup (Figure 6)(44, 45). Subsequently, interest has focused on the dynamics of α-CEHC excretion and a growing list of other minor metabolites; many questions, however, remain to be answered. For example, evidence from limited studies have reported that only a small fraction—as low as 5% of administered α-tocopherol—is recovered as α-CEHC in the urine (44, 46). Therefore, methods that can account for the full complement of biological metabolites are sought currently.

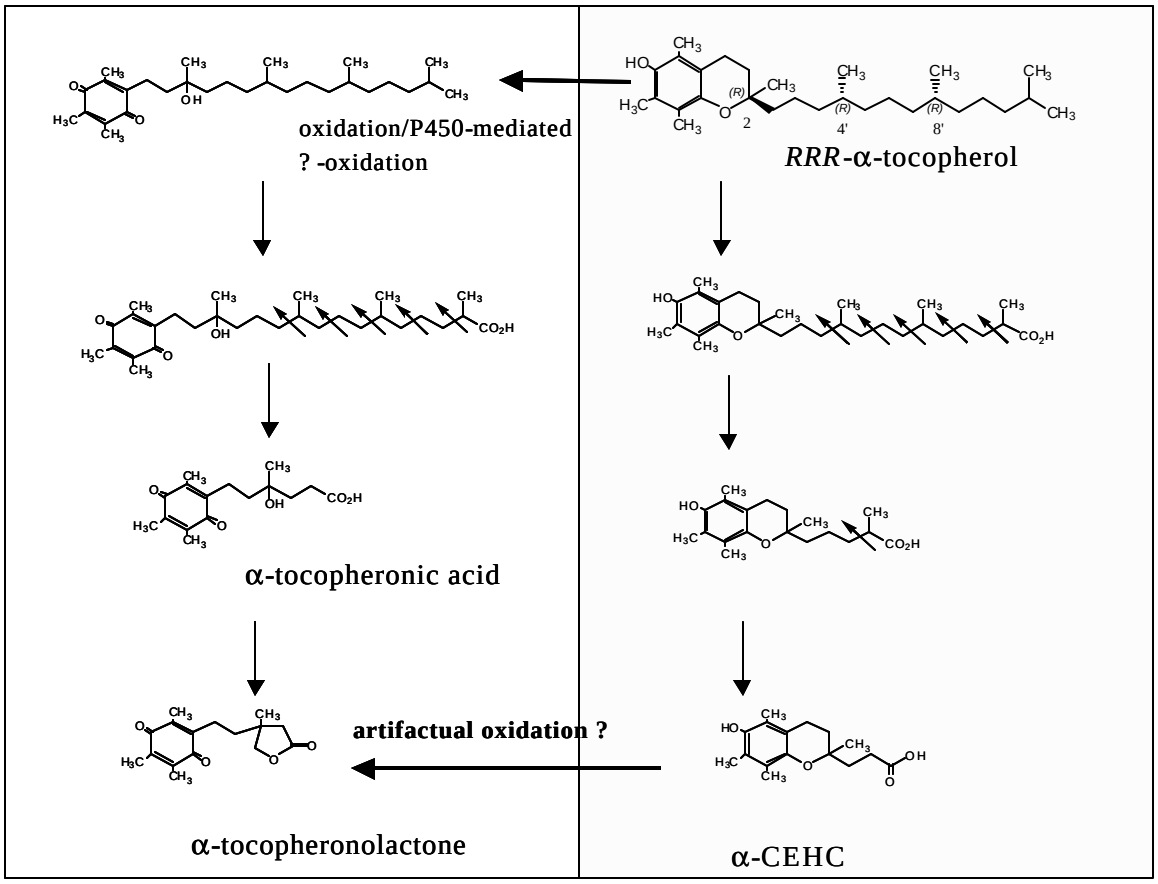


Figure 6*: Pathways of tocopherol metabolism. Current understanding states that participation as an antioxidant leads to the opening of the chroman ring and lactone products (left side), whereas an intact chroman ring (right side) is hypothesized to represent excretion of excess tocopherol. The simon metabolite, a-tocopheronolactone, appears to be a produced by artifactual oxidation of a-CEHC during the extraction procedure. (scheme adapted from Pope, 2001)*

Aspects of this issue were addressed in the aforementioned study of the individual who had consumed true tracer doses of natural and synthetic α-tocopherols. Within this study, the urinary metabolites were speciated. The procedure employed was that described by Lodge et al (46), involving deconjugation by glucuronidase treatment to, and partitioning of the metabolites, into ether. Urine samples were ' cold spiked' with the α-CEHC and 'Simon' metabolites at concentrations that would be easily viewed in the column effluent by UV/Vis absorbance. Similar to standard HPLC means of quantification by spectrometry, compound identification comes from the characteristic retention times of the known metabolites. In the described example, α-CEHC and the 'Simon' metabolites were available and added to the urine, prior to processing. An injection corresponding to ~ 80 ul of urine was then separated by reversed-phase HPLC with 20-30 sec eluent fraction collected and analyzed for ^{14}C by AMS.

In the adjoining radiochromatogram (Figure 7), peak concentrations of ^{14}C coincided with the α-CEHC standard. In contrast, little or no ^{14}C appeared at the characteristic elution time for the 'Simon' metabolites. This later result affirmed that the integrity of at least some of the metabolites, namely α-CEHC, was preserved in the extraction procedure. The activity of ^{14}C was not exclusive to the α-CEHC metabolites. Observing 'peak tops' in the radiochromatograms, a minimum of 9 chemically distinct metabolites carry the ^{14}C tag in both the natural and *synthetic* urine samples. Speculation as to the identity of these other peaks is beyond the scope of this presentation, but educated guesses can be made based upon literature information, *in vitro* experiments, and understanding of the chromatographic system. The *natural* and *synthetic* profiles present some observable differences: the peak after 8 minutes in the *synthetic* profile was absent in the *natural* profile, indicating differential pathways of catabolism for the natural and synthetic forms.

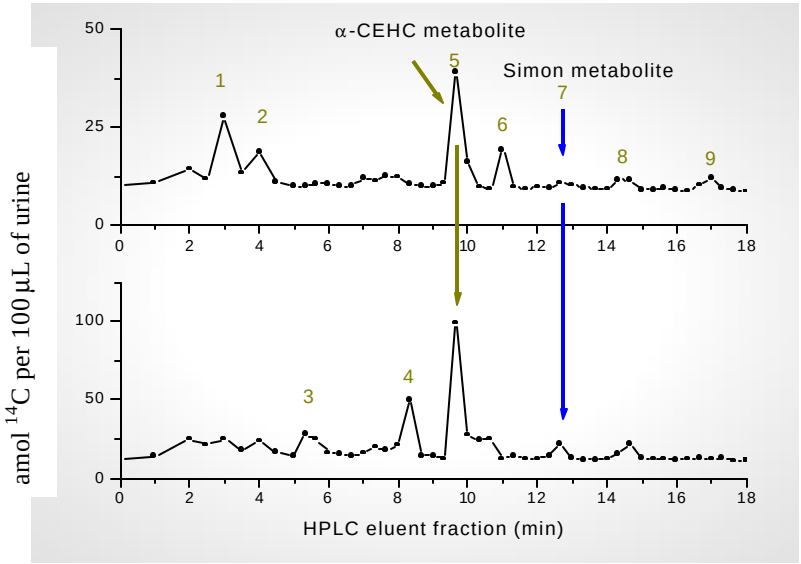


Figure 7: *^{14}C contents in HPLC eluents from human urine after consumption of RRR (top panel) and all-rac (bottom panel) a-tocopherol. Baseline is 9.0 amol ± 0.55 using a blank injection run. The limit of quantification is 1.6 amol per eluent fraction.*
a-CEHC is the single major metabolite in both tests, although at least 9 metabolites are indicated in the combined radiochromatograms

The limit of quantification (LOQ) for HPLC-AMS is determined by the average of the baseline fractions plus two standard deviations and is typically in the vicinity of 2-20 amol ^{14}C (36). Even lower levels of detection can be achieved through application of cleanup methods for removing other sources of biogenic carbon (lipid, proteins, etc) and higher specific activity compounds (but not higher radiative doses). HPLC fractions such as these have too little carbon for direct processing graphite, therefore 1 mg or more carbon is added to the dried eluents in the form of tributyrin.

Many factors can affect recovery from biological samples; in the case of reactive metabolites, the formation of artifacts can lead to spurious theories of metabolism. The ideal approach to sample preparation is to exclude the step altogether or "*dilute and shoot*" (47). It was hoped that multiple sector MS/MS techniques might achieve this possibility. However, this ideal remains to be realized, primarily due to problems and limitations associated with matrix-suppression of ionization or increased incidence of ion-molecule interactions. Similar concerns do not pertain to AMS-based method for the obvious reason that the sample is

converted to graphite prior to analysis and quantitation is based upon the ratio of the carbon isotopes. Therefore, minimal, or even no sample preparation affords an opportunity to view the metabolite profile in their native states. Metabolite profiling by direct injection of unprocessed urine has been demonstrated in other applications (29, 48).

b-Carotene: cooperative behavior

The pigments found in plants exercise vital functions of plant metabolism. Furthermore, they provide visual attraction in nature and for humans, provide us with nutrients. Indeed, many plant pigments perform essential roles human and animal health. An early record of the positive association between plant pigments and human health is found in 1919. In this record, Steenbock noted that yellow corn (*Zea mays* L.) and "yellow" vegetables, such as carrots (*Daucus carota* L.) and sweet potato (*Ipomoea batatas* L.) eliminated the symptoms of vitamin A deficiency in rats. Conversely, white corn and "white" vegetables, such as parsnip, (*Pastinoca sativa* L.), potato (*Solanum tuberosum* L.), and beets (*Beta vulgaris* L.) did not. An explanation of these observations was found when it was discovered that enzymes in the intestine and other parts of the human body enzymatically convert certain yellow colored carotene to vitamin A active compounds (49). Of these carotenes, ß-carotene is one of the most commonly consumed and easily metabolized forms of provitamin A pigments. In fact, it provides about 80% of the vitamin A needs globally. However, despite its abundance, Vitamin A deficiency is presently plaguing many developing nations, afflicting millions of children each year with xerophthalmia, blindness, or death.

ß-carotene's relationship to health may extend well beyond its function as a vitamin A source. Numerous studies have observed inverse correlations between plasma carotene concentrations and certain cancers. Based on these and other data, several large chemoprevention trials have been conducted over the last several decades. The results of these trials found supplemental ß-carotene to provide no benefit or, in fact, cause harm in smokers. In the aftermath of these studies, it has become clear that many fundamental aspects of β-carotene metabolism were poorly understood in humans. The observed toxicity of carotene has called into question the use of isolated phytochemicals at supraphysiological levels, as well as, strengthened the case for food-based approaches to the prevention of chronic disease.

There are several reasons why food could succeed where supplements fail. One hypothesis states that nutrients act cooperatively and the body is optimized towards utilizing these nutrients when given as complex mixtures and at dietary levels. Marked deviations from this balance may then lead to altered distributive kinetics, affecting the chemical and anatomical disposition of the nutrients. An example relevant to ß-carotene and AMS capabilities involves the effect of vitamin A status on ß-carotene metabolism. Although marginally tested, it is popularly suspected that vitamin A status inversely relates to the utilization of ß-carotene as a vitamin A source in humans (50). To test this hypothesis, at risk population of marginal vitamin A status, typically children, are selected and then fed ß-carotene. Individuals of low status will supposedly derive greater vitamin A benefit from the administered ß-carotene than those that who are vitamin A adequate (51). These studies, however, require the participation of experimental subjects of marginal nutrient status and such subjects are difficult to locate in industrialized countries. Moreover, to the extent that phytonutrition is often focused on optimal health in nutritionally replete population, rather than correcting deficiencies, it is of interest to have methods that are sensitive to subtle physiological changes in replete population.

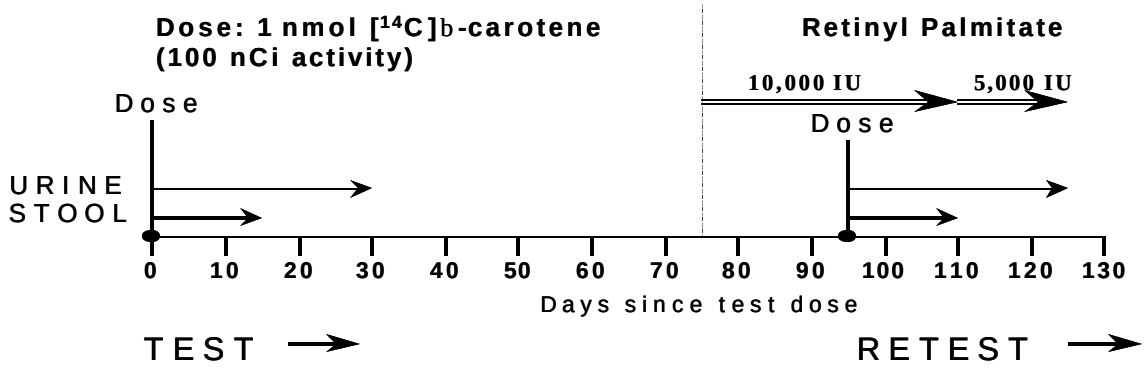


Figure 8*: Experimental design and time line. The experiment was designed to incorporate a test and retest period. In the test period, subjects began complete fecal and urine collection 24 h in advance of the dose and continued complete 24-h collections until day 16 and day 30, respectively. . The subjects were then given a 1 nmol dose of [^{14}C]b-carotene in an emulsified drink. Cumulative urine was collected for 30 d and cumulative stool for 17 d. . This was followed by a seven-week wash-out period. After that time, the administration of a second [^{14}C]b-carotene dose marked the beginning of the retest period. Three weeks prior to the start of the retest, subjects began consuming 10,000 IU (3000 mg RE) of vitamin A supplement daily and continued at that level until two-weeks after the retest dose administration. Then the supplement was continued at 5000 IU (1500 RE) until the completion of the testing.*

A recent study conducted at the University of California, Davis and Lawrence Livermore National Laboratory, highlights the unique opportunities that AMS provides in the study of replete populations where other biomarkers might respond to subtle changes in dietary intake. The effect of vitamin A supplements on the absorption and metabolic behavior of a physiological dose of ^{14}C-ß-carotene was investigated in test-retest format. Specific details of the dose time line are shown in Figure 8. Complete fecal and urine samples were collected for

14 d and 30 d, respectively. After the test, both subjects consumed daily supplements of 10,000IU retinyl palmitate for 21d to raise their vitamin A nutritional status. Both subjects then took a second and similar dose of ^{14}C-ß-carotene.

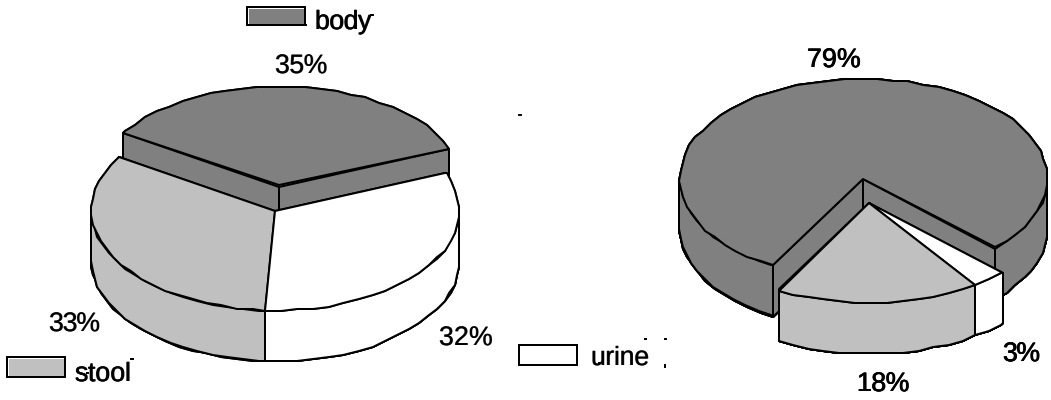


Figure 9: *Effects of vitamin A supplementation upon the disposition of a tracer dose of ^{14}C-ß-carotene in a longitudinal test in a female participant. The charts illustrate the dose disposition in excreta and subsequent body burden prior to (left) and following supplementation with vitamin A (right). Supplemental vitamin A resulted in increased absorption (stool) and retention (urine and body burden) of a tracer dose of ß-carotene in vitamin A replete participants.*

In the vitamin A adequate individual, the effect of supplemental dose in moderate excess of normal dietary levels (2 X RDA) might not be expected to substantially impact the utilization of ß-carotene. The results suggest otherwise and are illustrated in the adjoining chart (Figure 9) where the dose disposition 72 hours post-dose for both the test and retest periods is exhibited. Absorption, as assessed by the cumulative recovery of the ^{14}C label in the stools was raised by about 50% in both subjects. Conversely, the recovery of ^{14}C in the urine was reduced by 10-fold in both subjects. As a result, 72 hours after dosing, about 80% of the consumed vitamin A was retained in the body of the supplemented individuals, whereas only about 35% was retained in the untreated and same subjects.

These data suggest that raising the vitamin A status in already replete subjects can markedly raise absorption and subsequent retention of a physiological dose of β-carotene. Such data has important implications for recommendations regarding the consumption of β-carotene either in food-based or supplemental forms. Minor changes in the consumption of one nutrient can have additive, or even synergistic, effects on the utilization of a second nutrient, even in nutritionally replete populations that, by all definitions, are not lacking in either nutrient.

Additionally, these data illustrate the value of *non-invasive* collections, ie urine and stool for the assessment of phytonutrient metabolism. Because the dose input is known, a full

accounting of the ^{14}C is possible without the collection of blood specimens. Accordingly, the total of the body burden of the labeled compound is obtained. Such experimental models clearly represent an attractive method for assessing the biological variance in the absorption and retention characteristics of phytonutrients with specific analyte identification methods. This non-invasive feature, causing minimal discomfort in participants, would greatly facilitate population surveys into the utilization of selected phytonutrients.

Radiation Risk

When discussing the role of radiation in AMS, it is first important to discuss the role of radiation in the natural world. Our world is radioactive – when assessing the radiation risk in experimental subjects, one must incorporate voluntary and involuntary exposures presented by our environment, diet, and lifestyle choices. Natural radiation from cosmic rays and from naturally occurring radioisiotopes accounts for the vast majority of the total average effect dose. Indeed, approximately 81% is from natural sources of radiation, and of that, most is from radon. Every food has radionuclides which lead to the accumulation of radioisotopes with the body. The common radionuclides in food are radiocarbon (^{14}C), as well as potassium 40 (^{40}K), radium (^{226}Ra) and uranium (^{238}U) and their associated progeny. An average human body experiences about 500,000 radioactive disintegrations per minute due to these and other radionuclides. The integrated average exposure is about 3600 μSv, a Seivert being the deposited energy equivalent to a joule per kilogram.

It is important to consider the amount of ^{14}C that naturally occurs in the body. The human body is about 23% carbon. The natural background levels of ^{14}C are due to formation in the upper atmosphere (cosomgenic production) as well as contribution from anthropomorphic sources (the burning of fossil fuels and atmospheric testing of nuclear weapons) is 6.11 picoCi/g carbon (0.23 Bq). Accordingly, approximately 100 nanoCi (37 kBq) is in a 70 kg person. An average adult might consume 200 grams of carbon in a 24-hr period which would contain 2.1 nanoCi of ^{14}C. By contrast, a 'large' administered radio-dose in AMS experiments is 100 nanoCi. In the case of vitamins, this quantity was determined to obtain long-term kinetic information associated with the longer turnover times inherent to fat soluble vitamins (β-carotene $t_{1/2}$ ~40 d) and even retained water soluble forms (folate $t_{1/2}$ ~100 d). Experiments in ß-carotene and folic acid were conducted for greater than 200 days (26, 27). A majority of studies will be primarily interested in those events occurring within the period of absorption and distribution of a dose, therefore studies on the limited duration of several days can be conducted. Where long-term tracing is not necessary, doses of 10 nanoCuries or less suffice. In such cases, the amount of administered radiocarbon would be equal to that consumed in several meals. By this analogy, it is hard to imagine that low radiation dose AMS studies pose any quantifiable risk to the participant.

Labeling Strategies

Stable and radioisotopes of hydrogen and carbon are often incorporated into organic molecular structures to serve as isotopic tracers. However, despite the ingenuity of modern organic chemists, many natural products found in common foods or medicinal plants are not amenable

to isotopic synthesis due to their complex stereochemistry and distribution of unusual structures. For example, within the flavonoid family, proanthocyanidins (condensed tannins) are polymeric entities which constitute one of the most abundant polyphenols in the human diet. These compounds are responsible for the astringency, and to some extent the bitterness, of many fruits such as grapes, apples, and persimmons. They bring these same qualities to fruit-derived beverages, most notably wine. The molecular weight of proanthocyanidins can vary between 600-3000 (dimers), although molecular weights as great as 30,000 Da have been recorded. Such a variance clearly poses a daunting synthetic challenge.

As a more practical alternative to isotopic synthesis, labeled compounds can be obtained by the introduction of isotopically labeled precursors into various portals. Labels can be incorporated into intact plants, as well as excised tissues, cultured plant cells, algae or isolated enzymatic homogenates. Harvesting the natural capabilities of biosystems has several advantages. First, detailed knowledge of synthetic chemistry is unessential. Secondly, the appropriately selected organism will replicate the natural complexity of the desired analytes. Finally, there is minimal production of hazardous waste products pertinent to organic synthesis. (52).

The value of isotopes (stable or radioactive) as molecular tracers depends on the detection methods: isotope ratio mass spectometry (IRMS), nuclear magnetic resonance (NMR), decay production counting, or AMS. Furthermore, the final detection method will often dictate the level of sophistication employed in the labeling protocol. It is established that methods developed around the detection of stable isotopes generally require much higher levels of isotope incorporation into the molecule— to facilitate detection— than corresponding radiometric methods (particularly AMS).

One demonstration of biolabeling is that of carotene in the photosynthetic alga, *Dunaliella Salina* (Figure 10). This organism accumulates large amounts of ß-carotene in response to the stress of nutrient depletion and increased light intensity. As it is photosynthetic, it offers the opportunity of incorporating labeled carbon into its metabolic product by the feeding of labeled CO_2. Wilson and coworkers detailed the construction of a 4.5 liter photosynthetic bioreactor for the production of ^{13}C-labeled carotenes using a $^{13}CO_2$ precursor (53). The design of the reactor was optimized for the conservation of the label. This was achieved by using a gas recycling protocol during the feeding of the label into the system and a high level of incorporation of label into the ß-carotene target. Between 0.7 and 1.4 g/day of $NaH^{13}CO_3$ were fed for ~25 days in several experiments. Mass spectra data demonstrated high levels of incorporation of ^{13}C into the ß-carotene. In one experiment, the average molecular formula of the ß-carotene showed 30 of the possible 40 carbons in the molecule possessed the ^{13}C label. These high levels of label were deemed necessary for the efficient detection of ß-carotene in human tracer studies using traditional mass spectrometry. Parker demonstrated the value of highly labeled ß-carotene in human kinetic studies using GC-IRMS detection (54).

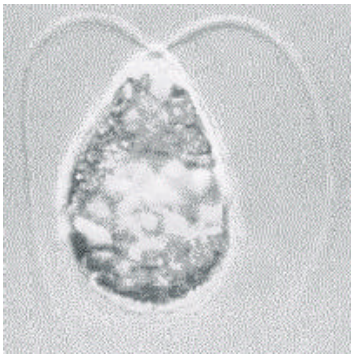


Figure 10*: Photo of Dunaliella Salina, a salt tolerant photosynthetic alga that makes copious quantities of all-trans and 9-cis b-carotene*.

Unlike the aforementioned example, the high sensitivity of AMS obviates the need for high levels of isotope incorporation (specific activity) or sophisticated labeled chambers. In one experiment (unpublished results), 3 milliCuries of NaH $^{14}CO_3$ was added to 50 ml culture of *D. Salina* grown in a 50 ml screw cap bottle. A light source was provided and the contents of the closed vessel were allowed to grow for 1 week. The final specific activity of the ß-carotene measured 30 Ci/mol, which corresponded to approximately 1-^{14}C atom per two molecules of β-carotene (By contrast, a perlabeled ^{14}C-ß-carotene, as used in Parker's study, would possess a specific activity of 2500 Ci/mol if ^{14}C were substituted for the ^{13}C nuclide). Nonetheless, even at these low levels of incorporation, a nanoCuried-sized dose could be traced in human plasma for > 200 days by AMS (27).

The incorporation of labeled CO_2 as a bicarbonate into *D. Salina* and other organisms represents a generic labeling strategy. A similar approach can be applied for the photosynthetic labeling of intact plants as well using atmospherically sealed plant chambers. An example of a chamber appropriate for radiolabeling with $^{14}CO_2$ is illustrated in Figure 11. This chamber ensures controlled cycles of irradiance, temperature and relative humidity while quantitatively containing the administered radiolabel. The system is primarily comprised of: a stainless steel chamber, a refrigerated water chiller, a water reservoir with pump, a 1000 Watt metal halide lamp suspended from an adjustable support arm, a control panel, a pressure relief system, and an on-time totalizer for administering CO_2.

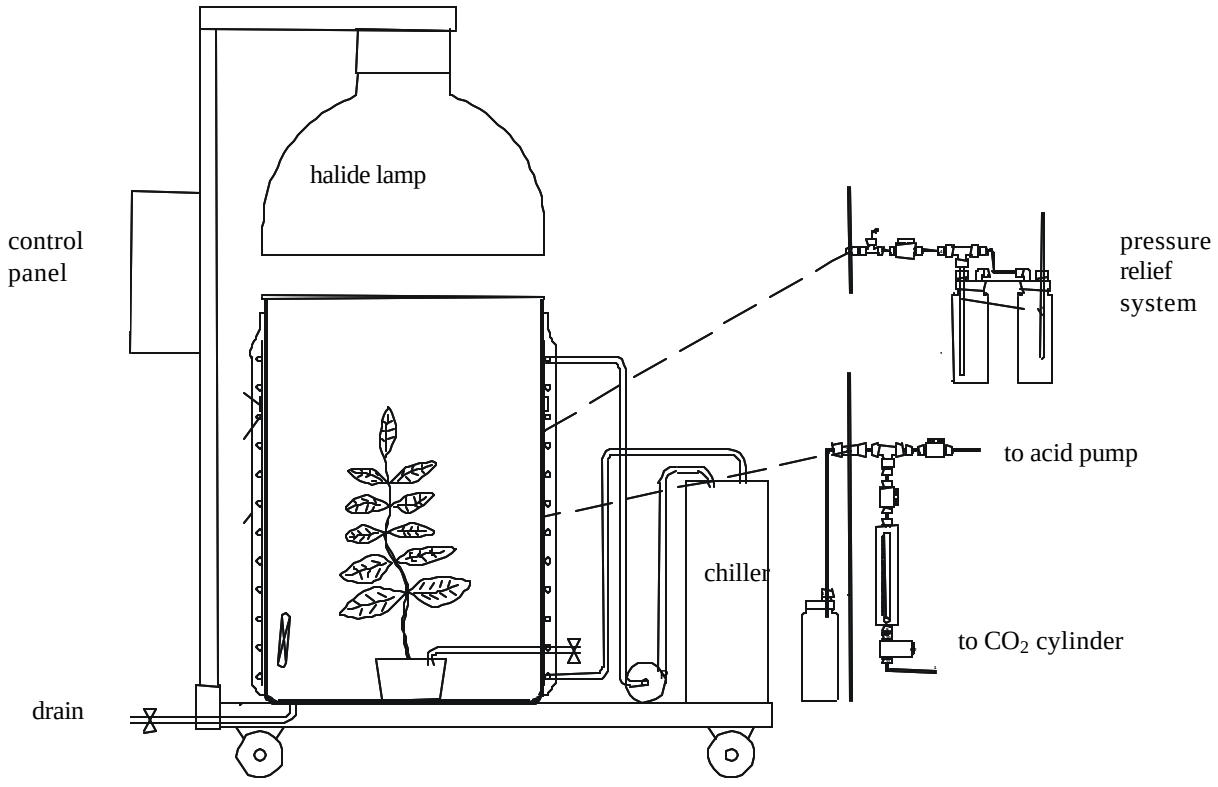


Figure 11: *Photosynthetic labeling chamber*

An application of the described isolation chamber is illustrated in a biolabeling study of common spinach (*Spinacia oleracea*). In this study, mature plants (30 days of age) were placed into the enclosed chamber. Exposure was initiated by adding 10 milliCi/d as a solution of $NaH^{14}CO_3$ to excess acid, which was repeated for an additional 4 days. Following the final exposure, the plants were maintained for 72 h in the chamber prior to harvesting to remove residual $^{14}CO_2$. The aerial parts of the plant were harvested and were extracted for ß-carotene and lutein: specific activities were 1.45 and 0.35 Ci/mol respectively, corresponding to 0.023 and 0.0056 ^{14}C atoms per molecule, respectively.

Within the interest of phytonutrition, there lies an increasing attention on the assessment of available nutrients from the intact plant source (as opposed to chemically isolated concentrates). Photosynthetic radiolabeling cannot be applied to address this interest as it would expose the participant to high, ethically unacceptable, levels of radiation. Stable isotope approaches circumvent this concern, however. These approaches are applicable when sufficiently high levels of isotope incorporation can be achieved to overcome sensitivity limitations. An important example of this concept is demonstrated in the biological tracing of deuterium labeled vitamin K (phylloquinone). In this example, broccoli grown hydroponically using 31 atom % deuterium hydroxide was fed to a single participant. The plasma concentration of the labeled vitamin K in the plasma could be traced for ~12 hours using GC/MS detection operated in the negative chemical ionization mode (55).

The concept of intrinsic labeling has been applied to the radiolabeling of folates in the pea plant (56). In these experiments, pea seeds were allowed to imbibe ^{14}C- *para*-aminobenzoic acid (pABA) prior to germination and vegetative growth (56) (Figure 12). Significant portions of the administered labeled appeared in the plant folate pools. While human testing was not the aim of this investigation, it does validate the general concept. The concept was also applied by our group for the specific purpose of assessing the availability of folates when

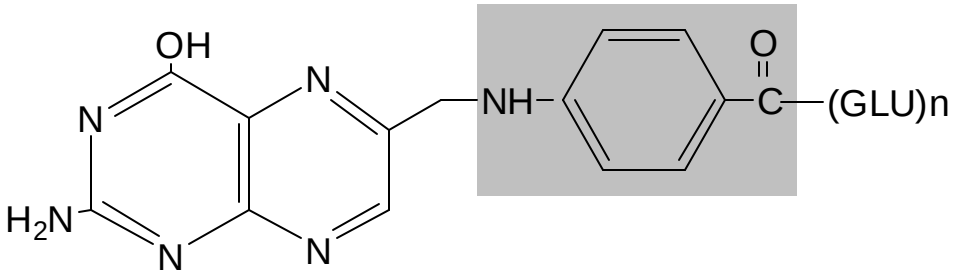


Figure 12*: Stylized representation of folic acid. Grey area represents moeity derived from pABA*

consumed by an intact plant. In this study, ^{14}C-pABA was administered to a mature kale plant (*Brassica oleracea acephala*) using the cotton-wick method. After uptake of the label, the plant was then allowed to grow for one week to promote translocation of the dose to the actively growing leaf tips, the area of most active respiration. This experiment was unsuccessful, however, as quantities of the administered label were not assimilated into the folate pools in the degree necessary to facilitate testing. Most of the activity, rather, resided with unmodified ^{14}C-pABA. Nonetheless, further experimentation with growth cycles and label deliver techniques should prove eventually successful. Upon surveying varying labeling

strategies, it is clear that AMS sensitivity will provide a most amenable approach for difficult to synthesize phytochemicals.

Conclusion

As public interest in phytonutrition continues to increase, the result will be an augmented demand for extensive phytochemical research. The fact that foods are inherently phytochemically complex dictates a need to apply scientific techniques, which can detect synergistic interaction among the many active principles and adjuvant substances in the plant, and furthermore, modify the activities of these components. As illustrated by the experiments discussed in this presentation, the advantages of AMS are unique and extensive. These advantages are best summarized by Dr. John Vogel, an originator of biological AMS experimentation: "AMS brings (at least) three advantages to biochemical tracing: high sensitivity for finding low probability events or for use of physiologic-sized doses; small sample sizes for painless biopsies or highly specific biochemical separations; and reduction of overall radioisotope exposures, inventories, and waste streams." AMS opens the door to increased phytochemical tracing in humans to obtain biochemical data concerning human health at dietary relevant levels of exposure. AMS, thus, obviates the need for uncertain extrapolations from animal models, which express marginal relevance to human metabolism. The unparalleled capabilities and benefits of AMS will undoubtedly establish this particular MS technique as an important analytical tool in phytochemical research.

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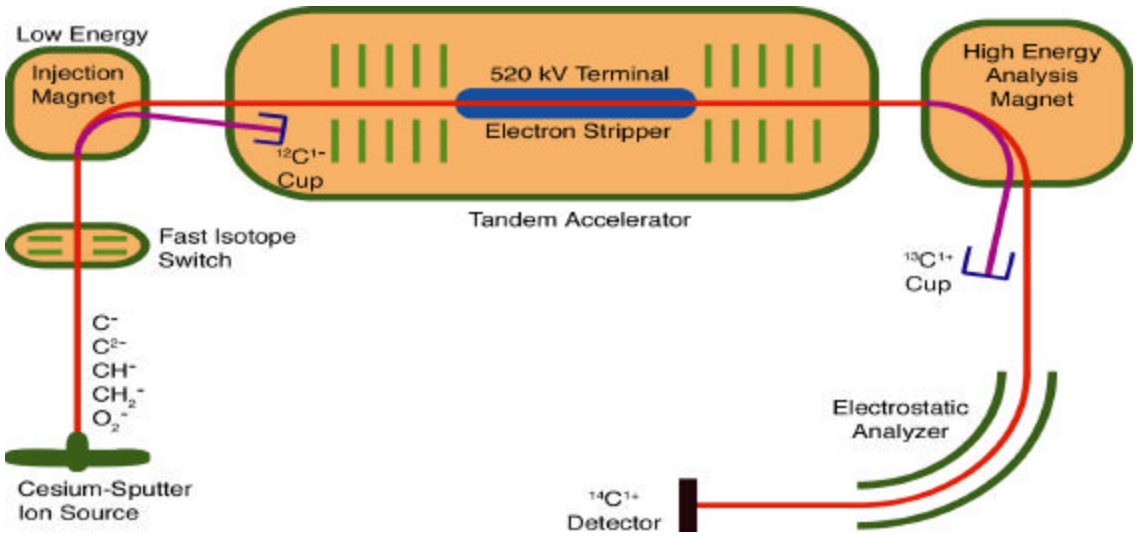
References

1. Grusak, M. A. *Curr Opin Biotechnol* 13 (2002) 508-11.
2. Potrykus, I. *Plant Physiol* 125 (2001) 1157-61.
3. Farnham, M. W., Simon, P. W., and Stommel, J. R. *Nutr Rev* 57 (1999) S19-26.
4. Mullineaux, P. M., and Creissen, G. P. *Biochem Soc Trans* 24 (1996) 829-35.
5. Block, G., Norkus, E., Hudes, M., Mandel, S., and Helzlsouer, K. *Am J Epidemiol* 154 (2001) 1113-8.
6. Halsted, C. H. *Am J Clin Nutr* 77 (2003) 1001S-1007S.
7. Albanes, D. *Am J Clin Nutr* 69 (1999) 1345S-1350S.
8. Omenn, G. S. *Annu Rev Public Health* 19 (1998) 73-99.
9. Van Pelt, C. K., Haggarty, P., and Brenna, J. T. *Anal Chem* 70 (1998) 4369-75.
10. Mellon, F. A., Bennett, R. N., Holst, B., and Williamson, G. *Anal Biochem* 306 (2002) 83-91.
11. Sumner, L. W., Mendes, P., and Dixon, R. A. *Phytochem* 62 (2003) 817-36.
12. Goodacre, R., York, E. V., Heald, J. K., and Scott, I. M. *Phytochem* 62 (2003) 859-63.
13. Hopfgartner, G., and Bourgogne, E. *Mass Spectrom Rev* 22 (2003) 195-214.
14. Vogel, J. S., Turteltaub, K. W., Finkel, R., and Nelson, D. E. *Anal Chem* 67 (1995) 353A-359A.
15. Vogel, J. S., and Turteltaub, K. W. *Adv Exp Med Biol* 445 (1998) 397-410.
16. Jackson, G. S. W., C.;Elmore, D. *Nut Res Rev* 14 (2001) 317-334.
17. Mous, D. J. W. P., K.H.; Fokker, W.; Van den Broek, R.;Koopmans, R.B. *Nuc instr & meth* B123 (1997) 159-162.
18. Chiarappa-Zucca, M. L., Dingley, K. H., Roberts, M. L., Velsko, C. A., and Love, A. H. *Anal Chem* 74 (2002) 6285-90.
19. Dingley, K. H., Roberts, M. L., Velsko, C. A., and Turteltaub, K. W. *Chem Res Toxicol* 11 (1998) 1217-22.
20. Mellon, F. A. S., B. (1996) Stable Isooptes in Human Nutrition, Academic Press, London.
21. Goodman, D. S., Blomstrand, R., Werner, B., Huang, H. S., and Shiratori, T. *Jf Clinl Invest* 45 (1966) 1615-1623.
22. Gove, H. E. (1999) From Hiroshima to the Iceman, Institute of Physics Publishing, Bristol, UK.
23. Turteltaub, K. W., Felton, J. S., Gledhill, B. L., Vogel, J. S., Southon, J. R., Caffee, M. W., Finkel, R. C., Nelson, D. E., Proctor, I. D., and Davis, J. C. *Proc Natl Acad Sci U S A* 87 (1990) 5288-92.
24. Turteltaub, K. W., Vogel, J. S., Frantz, C., Buonarati, M. H., and Felton, J. S. *Environ Health Perspect* 99 (1993) 183-6.
25. Turteltaub, K. W., Vogel, J. S., Frantz, C. E., and Fultz, E. *IARC Sci Publ* (1993) 293-301.
26. Clifford, A. J., Arjomand, A., Dueker, S. R., Schneider, P. D., Buchholz, B. A., and Vogel, J. S. *Adv Exp Med Biol* 445 (1998) 239-51.
27. Dueker, S. R., Lin, Y., Buchholz, B. A., Schneider, P. D., Lame, M. W., Segall, H. J., Vogel, J. S., and Clifford, A. J. *J Lipid Res* 41 (2000) 1790-800.
28. Stenstrom, K., Leide-Svegborn, S., Erlandsson, B., Hellborg, R., Mattsson, S., Nilsson, L. E., Nosslin, B., Skog, G., and Wiebert, A. *Appl Radiat Isot* 47 (1996) 417-22.
29. Garner, R. C., Goris, I., Laenen, A. A., Vanhoutte, E., Meuldermans, W., Gregory, S., Garner, J. V., Leong, D., Whattam, M., Calam, A., and Snel, C. A. *Drug Metab Dispos* 30 (2002) 823-30.
30. Hoppe, P. P., and Krennrich, G. *Eur J Nutr* 39 (2000) 183-93.
31. Burton, G. W., Traber, M. G., Acuff, R. V., Walters, D. N., Kayden, H., Hughes, L., and Ingold, K. U. *Am J Clin Nutr* 67 (1998) 669-84.
32. Traber, M. G., Burton, G. W., Ingold, K. U., and Kayden, H. J. *J Lipid Res* 31 (1990) 675-85.
33. Lauridsen, C., Leonard, S. W., Griffin, D. A., Liebler, D. C., McClure, T. D., and Traber, M. G. *Anal Biochem* 289 (2001) 89-95.
34. Cohn, W. *Eur J Clin Nutr* 51 (1997) S80-5.
35. Sato, Y., Hagiwara, K., Arai, H., and Inoue, K. *FEBS Lett* 288 (1991) 41-5.
36. Buchholz, B. A. D., S.R.; Lin, Y.; Clifford, A. J.;Vogel, J. S. *Nuc Inst Meth Phy Res B* 172 (2000) 910-914.
37. Gilman, S. D., Gee, S. J., Hammock, B. D., Vogel, J. S., Haack, K., Buchholz, B. A., Freeman, S. P., Wester, R. C., Hui, X., and Maibach, H. I. *Anal Chem* 70 (1998) 3463-9.
38. Brigelius-Flohe, R. *Biochem Biophys Res Commun* 305 (2003) 737-40.

39. Sontag, T. J., and Parker, R. S. *J Biol Chem* 277 (2002) 25290-6.
40. Parker, R. S., Sontag, T. J., and Swanson, J. E. *Biochem Biophys Res Commun* 277 (2000) 531-4.
41. Pope, S. A., Burtin, G. E., Clayton, P. T., Madge, D. J., and Muller, D. P. *Free Radic Biol Med* 33 (2002) 807-17.
42. Brigelius-Flohe, R., Kelly, F. J., Salonen, J. T., Neuzil, J., Zingg, J. M., and Azzi, A. *Am J Clin Nutr* 76 (2002) 703-16.
43. Simon, E. E., A.; Sundheim, L.;Milhorat, A. II *Journal of Biological Chemistry* 221 (1956) 807-817.
44. Schultz, M., Leist, M., Petrzika, M., Gassmann, B., and Brigelius-Flohe, R. *Am J Clin Nutr* 62 (1995) 1527S-1534S.
45. Schultz, M., Leist, M., Elsner, A., and Brigelius-Flohe, R. *Methods Enzymol* 282 (1997) 297-310.
46. Lodge, J. K., Traber, M. G., Elsner, A., and Brigelius-Flohe, R. *J Lipid Res* 41 (2000) 148-54.
47. Henion, J., Brewer, E., and Rule, G. *Anal Chem* 70 (1998) 650A-656A.
48. Buchholz, B. A., Fultz, E., Haack, K. W., Vogel, J. S., Gilman, S. D., Gee, S. J., Hammock, B. D., Hui, X., Wester, R. C., and Maibach, H. I. *Anal Chem* 71 (1999) 3519-25.
49. Olson, J. A., and Hayaishi, O. *Proc Natl Acad Sci U S A* 54 (1965) 1364-70.
50. Solomons, N. W., and Bulux, J. *Ann N Y Acad Sci* 691 (1993) 96-109.
51. Van Lieshout, M., West, C. E., and Van Breemen, R. B. *Am J Clin Nutr* 77 (2003) 12-28.
52. Molyneux, R. J. *J Agric Food Chem* 50 (2002) 6939-42.
53. Wilson, P. D. G. H., M.G.;Waspe, C.R.;Steer, D.C.;Wilson, D.R. *Biotehnology Letters* 19 (1997) 401-405.
54. Parker, R. S., Brenna, J. T., Swanson, J. E., Goodman, K. J., and Marmor, B. *Methods Enzymol* 282 (1997) 130-40.
55. Dolnikowski, G. G., Sun, Z., Grusak, M. A., Peterson, J. W., and Booth, S. L. *J Nutr Biochem* 13 (2002) 168-174.
56. Cossins, E. A., and Chen, L. *Phytochem* 45 (1997) 437-52.

Figure 1. *Popular conceptions of foods have transformed substantially over the centuries. Now esteemed as a rich source of lycopene, to medieval Europeans the tomato had a baleful reputation*



Schematic of compact bio-AMS system at LLNL (Courtesy of Bruce Buchholz, LLNL)

Figure 2. *Biological samples for ^{14}C-AMS are typically combusted to CO_2 and then reduced to graphite. These samples are then bombarded by 3-10 keV Cs ions in a cesium-sputter ion source. This process physically knocks atoms and molecules out of the sample and contributes an electron to a fraction of the ejected particles, forming negative elemental or molecular ions. The production of negative ions removes the primary isobaric interference for radiocarbon, ^{14}N, since nitrogen does not form a stable negative ion. Singly ionized negative ions are accelerated to 40 kEV and then filtered by a low energy mass spectrometer that alternately switches between ions of mass about ^{14}C, i.e. ^{14}C, ^{13}CH, and $^{12}CH_2$ and mass 13, for the stable isotope measurements, into the accelerator. The field in the injection magnet is held constant and the isotopes are changed by quickly switching the energy (voltage) of the incoming ion beam. Negative ions are accelerated through 520 kV in the first stage of a tandem accelerator. At the end of this first acceleration stage these ions pass through an electron stripper, a small volume of ~45mT gas constrained by differential pumping. Here, particles undergo collisions, losing valence electrons. Depending on the accelerator potential, charge states of +1 to +4 are created. These positive ions then accelerate away from the positive potential to ground potential in the second half of the accelerator. The loss of electrons in the collision cells destroys all molecules, leaving only nuclear ions at relatively high energies (1.04 MeV for C). Positive ions exiting the accelerator are analyzed with a magnet and an electrostatic analyzer. An off-axis Faraday cup measures the $^{13}C^{1+}$ current after the analyzing magnet. A solid state particle detector counts individual $^{14}C^{1+}$ ions. The footprint of the compact Bio-AMS system is approximately 50 m^2.*

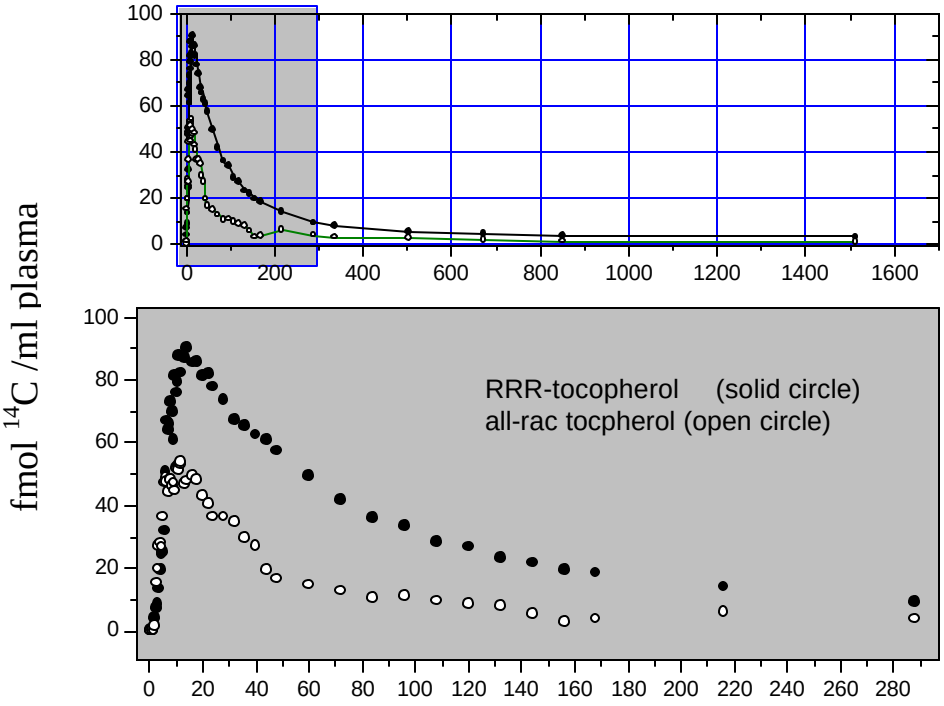


Figure 3*: Two ^{14}C doses were given in a longitudinal design in a single male volunteer: The first dose consisted of the single RRR isomer (RRR 3.7 kBq (100 nCi) , 0.85 ug). Three months later a similarly sized dose of the all-rac mixture was administered. Both doses were given with cup of whole milk (10 g fat) to facilitate absorption. Dose masses were 10,000 to 125,000 times less than similar studies using deuterium labeled isotopes with GC/MS or LC/MS detection.*

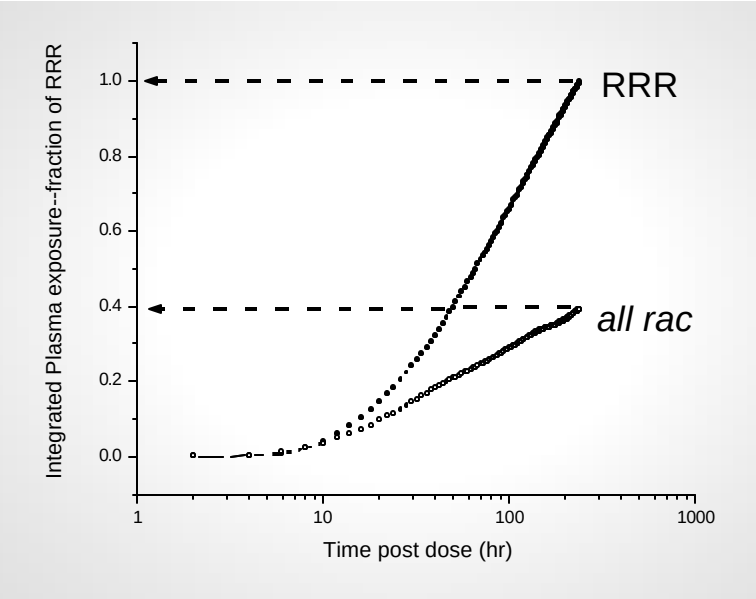


Figure 4*: Total plasma exposure to ^{14}C following administration of equivalent amount of natural (RRR) and synthetic (all rac)-a-tocopherol out to 250 hr post dose. A value of 1 is assigned to the RRR form. After 250 hr, the relative exposure to the synthetic form was about 40% that of the natural a-tocopherol.*

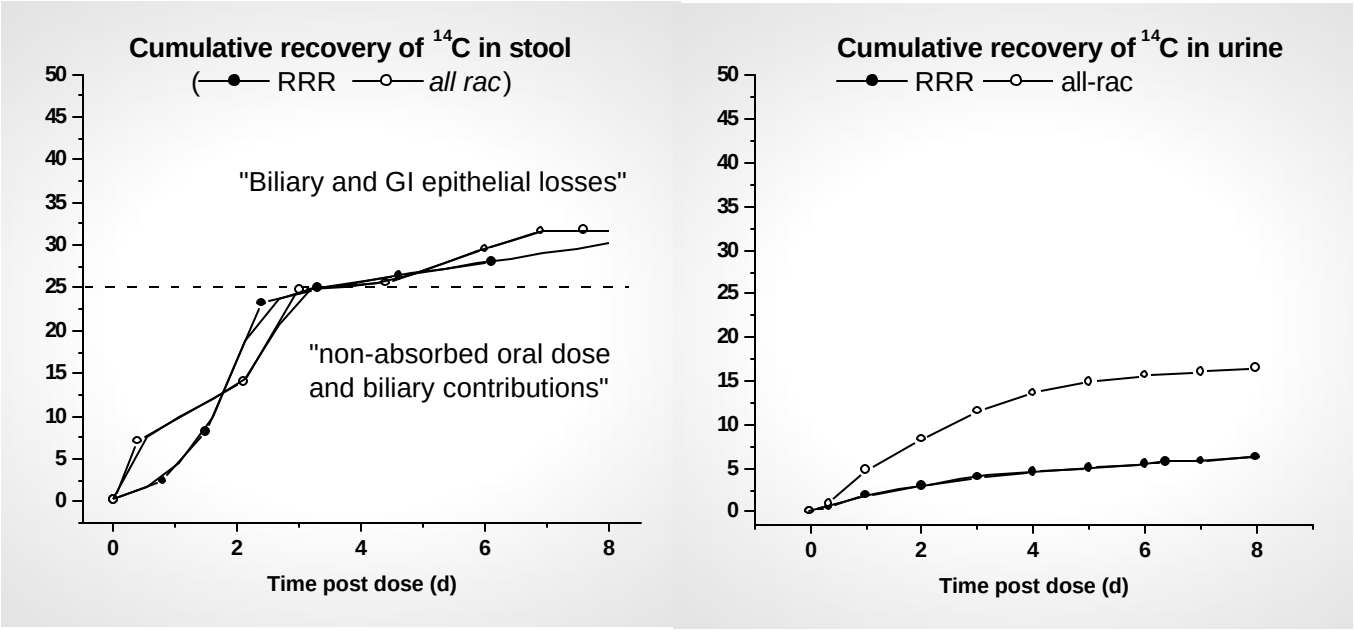


Figure 5*: Recovery of ^{14}C in urine (left) and stool following the two oral doses of labeled tocopherol. Intestinal absorption was equivalent for the two forms. Postabsorptively, the synthetic all-rac form underwent more rapid elimination via the urinary route. After 8 days, approximately 50% of the all rac, and 65% of the RRR were still present in the body.*

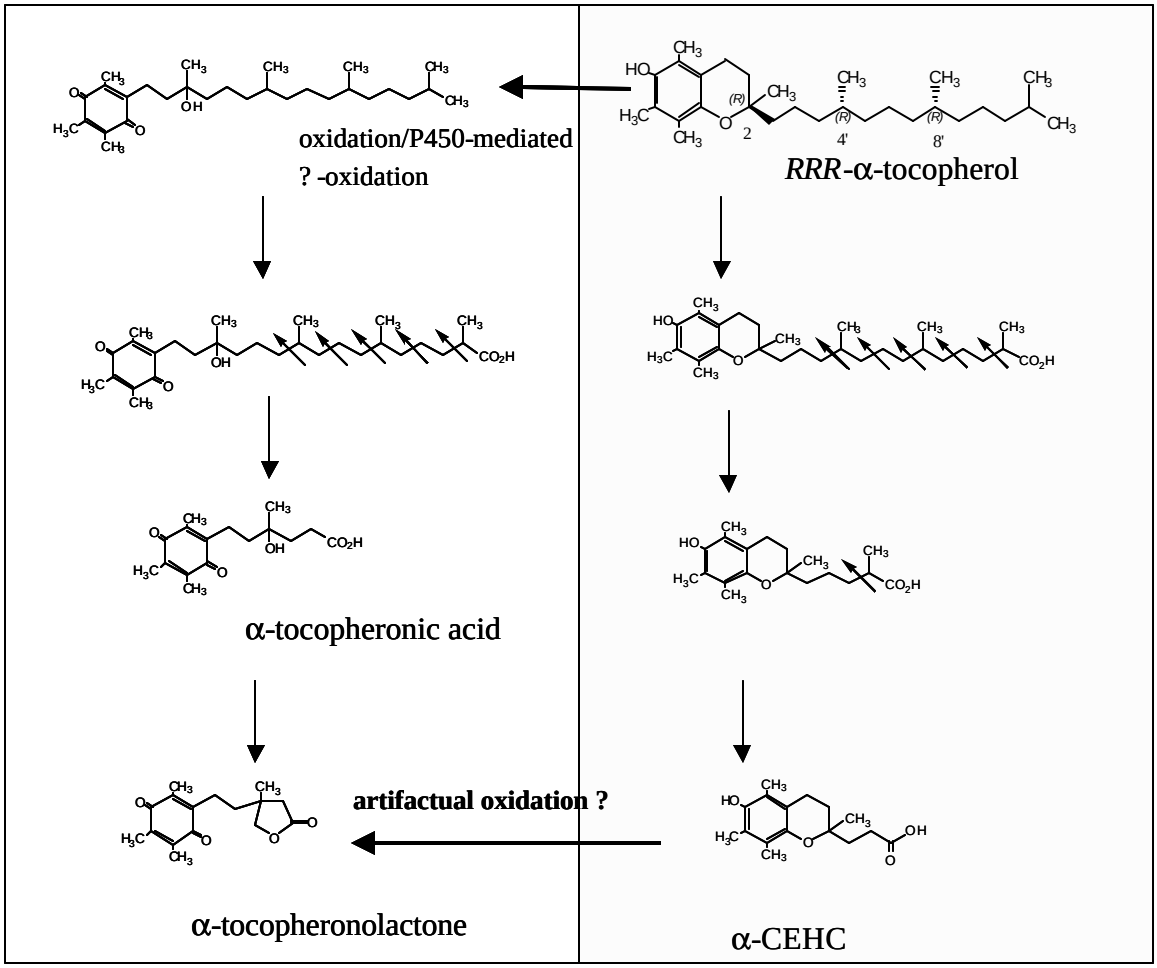
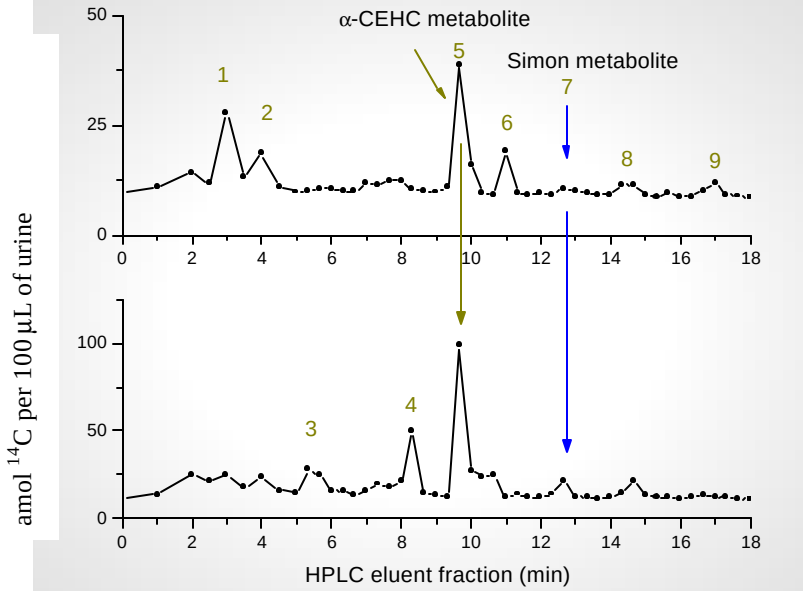


Figure 6*: Pathways of tocopherol metabolism. Current understanding states that participation as an antioxidant leads to the opening of the chroman ring and lactone products (left side), whereas an intact chroman ring (right side) is hypothesized to represent excretion of excess tocopherol. The simon metabolite, a-tocopheronolactone, appears to be a produced by artifactual oxidation of a-CEHC during the extraction procedure. (scheme adapted from Pope et al, 2001)*

Figure 7: *^{14}C contents in HPLC eluents from human urine after consumption of RRR (top panel) and all-rac (bottom panel) a-tocopherol. Baseline is 9.0 amol ± 0.55 using a blank injection run. The limit of quantification is 1.6 amol per eluent fraction.*
a-CHEC is the single major metabolite in both tests, although at least 9 metabolites are indicated in the combined radiochromatograms



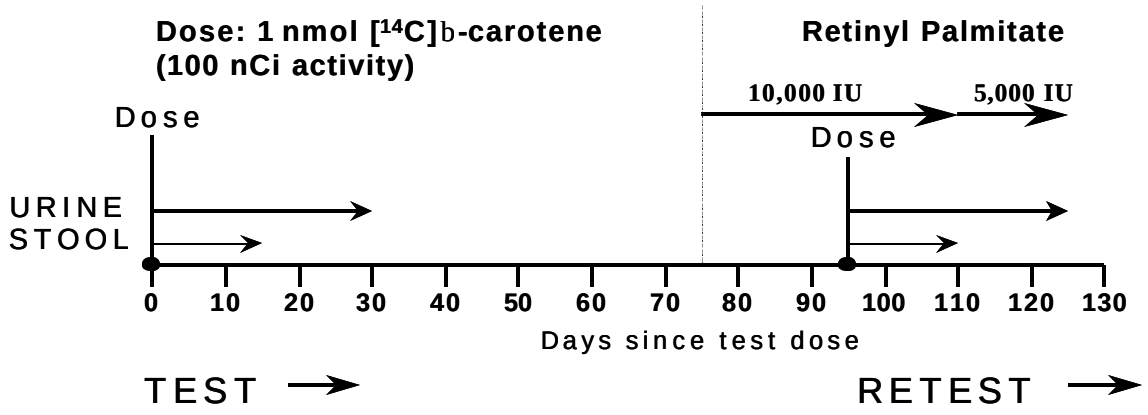


Figure 8*: Experimental design and time line. The experiment was designed to incorporate a test and retest period. In the test period, subjects began complete fecal and urine collection 24 h in advance of the dose and continued complete 24-h collections until day 16 and day 30, respectively. . The subjects were then given a 1 nmol dose of [^{14}C] b-carotene in an emulsified drink. Cumulative urine was collected for 30 d and cumulative stool for 17 d. . This was followed by a seven-week wash-out period. After that time, the administration of a second [^{14}C] b-carotene dose marked the beginning of the retest period. Three weeks prior to the start of the retest, subjects began consuming 10,000 IU (3000 mg RE) of vitamin A supplement daily and continued at that level until two-weeks after the retest dose administration. Then the supplement was continued at 5000 IU (1500 RE) until the completion of the testing.*

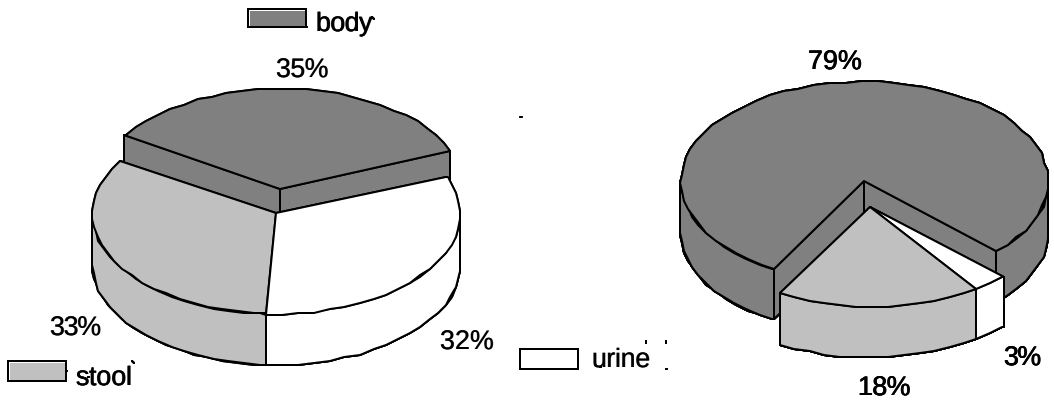


Figure 9: *Effects of vitamin A supplementation upon the disposition of a tracer dose of ^{14}C-ß-carotene in a longitudinal test in a female participant. The charts illustrate the dose disposition in excreta and subsequent body burden prior to (left) and following supplementation with vitamin A (right). Supplemental vitamin A resulted in increased absorption (stool) and retention (urine and body burden) of a tracer dose of ß-carotene in vitamin A replete participants.*

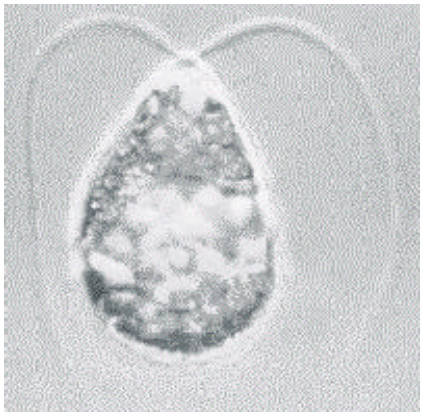


Figure 10*: Photo of Dunaliella Salina, a salt tolerant photosynthetic alga that makes copious quantities of all-trans and 9-cis b-carotene*.

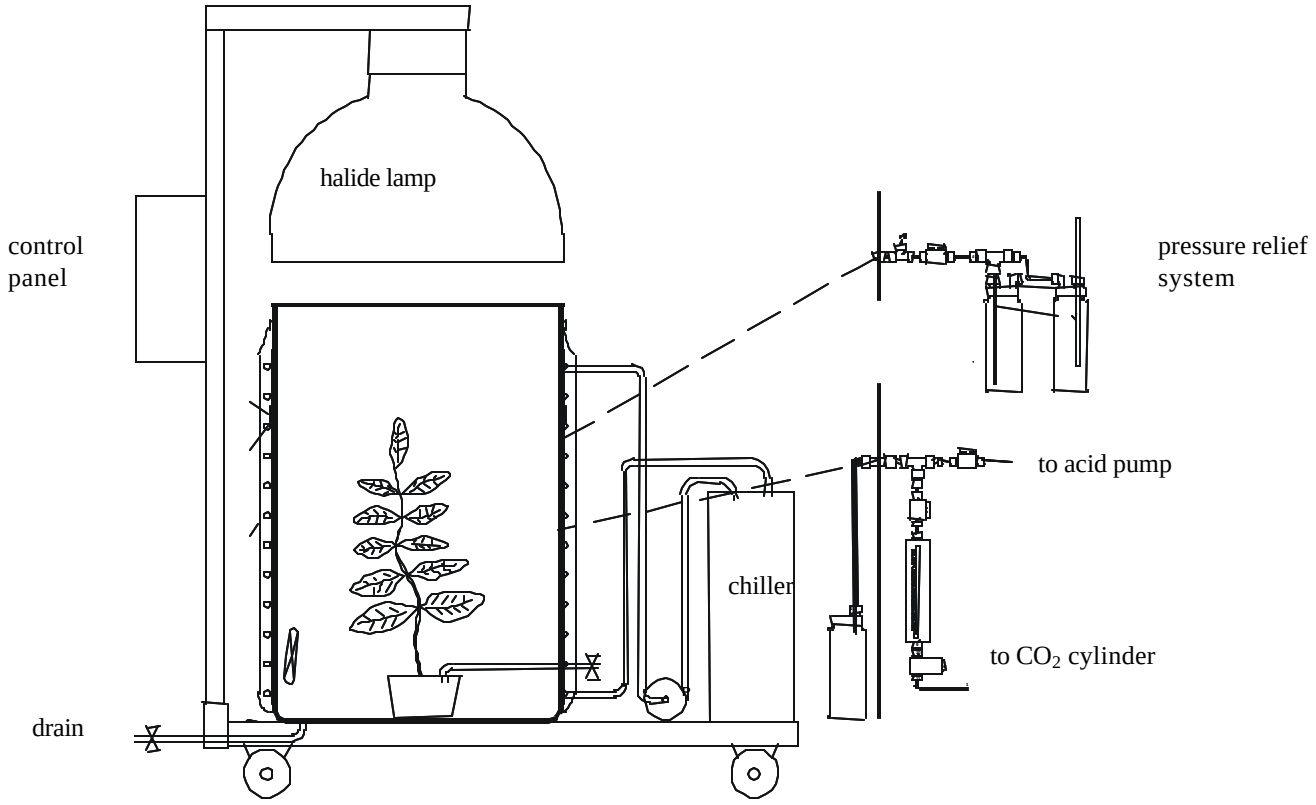


Figure 11: *Photosynthetic labeling chamber*

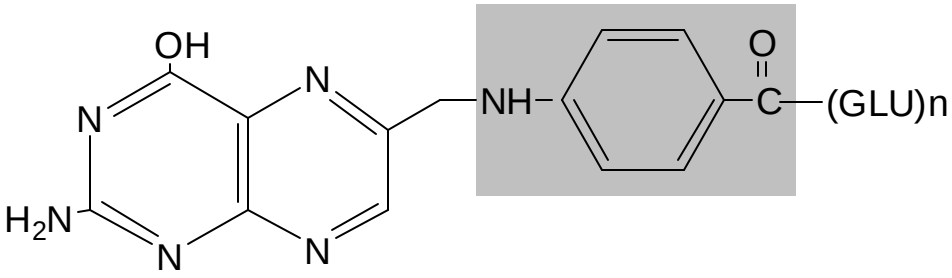


Figure 12*: Stylized representation of folic acid. Grey area represents moeity derived from pABA*